Quantitative Estimation of Bile Acid Conjugates in Human Bile Using HPLC

by

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ABSTRACT

QUANTITATIVE ESTIMATION OF BILE ACID CONJUGATES IN HUMAN BILE USING HPLC.

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An optimized reversed phase high performance liquid chromatographic method for the simultaneous analysis of the 10 major bile acid conjugates present in human bile is described. A one-step sample purification using solid phase extraction with Maxi-Clean® C-18 cartridges was applied. The reversed phase C-18 analytical column, an isocratic mobile phase with a constant flow rate (1.5 mL/minute), and UV detection at 215 nm were used. The original method of calculation using an internal standard was applied to the recovery study and to the quantitative estimation of the bile acid content of human bile. An efficient chromatographic resolution of the 10 bile acid conjugates was obtained by modification of the mobile phase composition to 65% methanol and 35% aqueous phosphate buffer (v/v) adjusted to pH 4.10, while preserving a relatively short chromatographic analysis of 45 minutes. The method was found to be reliable and convenient for the evaluation of conjugated bile acid in human bile.

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CHAPTER ONE

INTRODUCTION

Bile Acids:

Bile acids are the major metabolites from cholesterol disposal and are formed in the liver by action of the cytochrome P-450 oxidative pathway. The purpose of this oxidative pathway is to give the body a mechanism allowing non-polar substances that cannot be disposed of in any other manner to be partially polarized by the addition of oxygen as hydroxyl units. Such polarization is the body's attempt to make these non-polar materials more water soluble. This is particularly true for cholesterol since the alkane-like phenanthrene core of the molecule cannot be metabolized by the body, and remains intact throughout its metabolism. Adding hydroxyl groups to non-polar molecules greatly improves their water solubility by permitting hydrogen-bond interactions with water. As will be seen later in this discussion, the body makes further attempts to improve the solubility of non-polar materials that have been oxidized by the cytochrome P-450 pathway by further conjugation of the metabolites to a variety of charged molecules such as sulfate, glycine, or taurine.

The polarization of the non-polar molecules generally turns them into compounds that behave as detergents. This leads to an important additional role of bile acids in the body, besides disposal of cholesterol. They are also important detergents in the digestive tract that facilitate the digestion of lipids. As such, they are excreted into the digestive tract through the biliary system.

Bile acids may be classified as either primary and secondary depending upon the catabolic pathway that they have taken. Primary bile acids have been excreted directly from the liver after synthesis from cholesterol, while secondary bile acids have been reabsorbed by the body through the hepatobiliary blood system after primary bile acids

have been further metabolized by intestinal bacteria. Secondary bile acids are re-excreted in the bile after absorption. The most abundant bile acids in human metabolism are cholic (3,7,12-trihydroxycholanic), chenodeoxycholic (3,7,-dihydroxycholanic), and deoxycholic (3,12-dihydroxycholanic) acids. Cholic and chenodeoxycholic acids are primary bile acids and deoxycholic acid is a secondary bile acid.

In normal metabolism, excreted bile acids exist mainly as conjugates with amino acids glycine or taurine, with the glycine conjugates usually predominating. This process occurs in the liver and, as stated earlier, is a further attempt of the body to solubilize the non-polar cholesterol metabolites. The conjugated bile acids are stored in the gallbladder and are subsequently secreted into the intestine through the bile duct. The general function of the bile acids in the intestine is to help to emulsify fats, oils, and fatty acids. By this action they facilitate both the contact of emulsified fats with enzymes catalyzing hydrolysis and the absorption of the last through the intestinal wall (1,2). Table 1 represents the ten most common conjugated bile acids and the structures of some bile acids can be seen in Figure 1.

Table 1

Conjugated bile acids

Name	Class	Abbreviation
Glycocholic	Primary	GCA
Taurocholic	Primary	TCA
Glycodeoxycholic	Secondary	GDCA
Taurodeoxycholic	Secondary	TDCA
Glycochenodeoxycholic	Primary	GCDA
Taurochenodeoxycholic	Primary	TCDA
Glycoursodeoxycholic	Secondary	GUDA
Tauroursodeoxycholic	Secondary	TUDA
Glycolithocholic	Secondary	GLCA
Taurolithocholic	Secondary	TLCA

In the systemic circulation of a healthy human, bile acids of any form are found only in trace amounts. However, individuals afflicted with liver diseases and/or other related intestinal disorders may have the level and pattern of serum bile acids changed. The disturbances in synthesis and clearance of bile acids by the liver with following absorption in the intestine are the cause of this change. Such an abnormality can be detected with bile analysis and used for the diagnosis and treatment of patients (55-58). High-performance liquid chromatography (HPLC) has been widely used in bile analysis and is especially useful in simultaneous determination of the individual bile acids in biomedical samples (3).

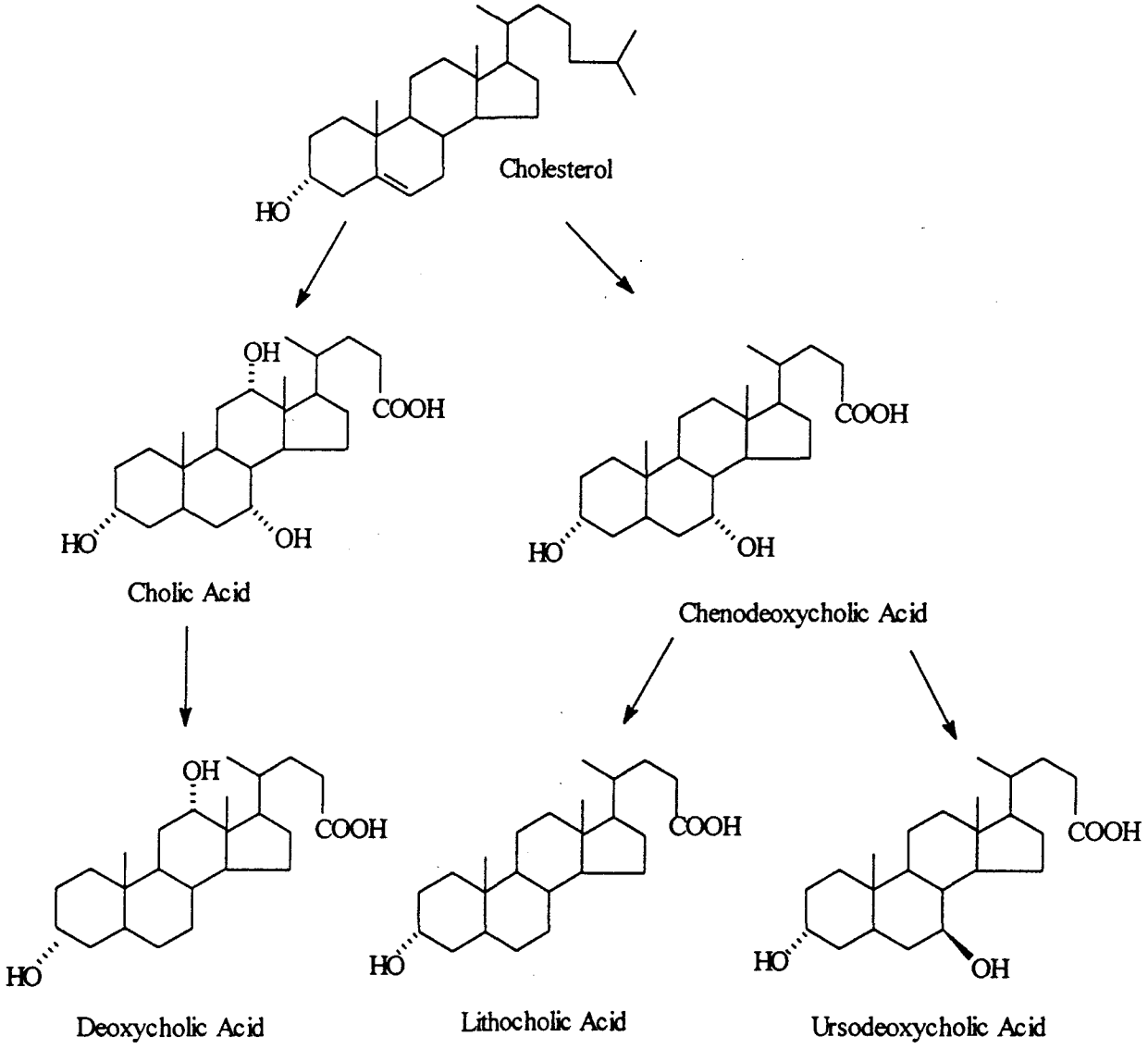


Fig. 1. Structures of Bile Acid

Bile acids are weak acids whose acidity is determined by their nature (i.e., the presence of carboxylic acid or sulfonic acid groups and/or the number of hydroxyls that are incorporated into their structure). Like other weak acids, they are ionized. The tendency to ionize or the strength of the acid is indicated by its pK_a value. The pK_a values of the free bile acids and of the glycine conjugates are in the range of 5.0 to 6.5 and 4.0 to 5.0, respectively. The pK_a values of the taurine conjugate are difficult to predict due to the relatively strong acidic nature of the conjugated taurine. However, it can be estimate from the pK_a value for free taurine, which is in the range of 1.5 to 1.5. Taurine conjugates, therefore, have a greater tendency to ionize and are more acidic than both the unconjugated (free) bile acids and the glycine conjugates.

The percent ionization of bile acids is dependent upon the solution pH value. The higher the pH, the larger the overall percentage of ionization of bile acids. In general, the free bile acids become protonated around pH 9.0, the glycine conjugates around pH 8.0, and the taurine conjugates around pH 3 or 4. In a normal individual the bile acids are thus mostly ionized at physiological pH (1).

The Retention Behavior of Conjugated Bile Acids in Reversed Phase HPLC:

The retention behavior of the conjugated bile acids in reversed phase HPLC (RP-HPLC) correlates with their acidity and was found to be intimately tied to the pK_a values of the conjugated glycine and taurine groups. In RP-HPLC, the ratio of the capacity factors of the glycine conjugated bile acids to those of the taurine conjugated bile acids is called the conjugation selectivity and is given by the following equation (59).

$$\alpha\,(G/T) = k'(G)/k'(T)$$

Here $\alpha\,(G/T)$ is the correlation selectivity and $k'(G)$ and $k'(T)$ are the capacity factors of the glycine and taurine conjugates, respectively.

It has been found in RP-HPLC of the 10 conjugated bile acids that for different methanol concentrations, but at fixed proportions of methanol (80, 75, 70, 65 %), that the

selectivity factors between the five glycine and taurine conjugates are identical. It has been also found, despite a considerable variation in retention for each pair of glycine and taurine conjugates using different octadecyl (C-*18)* stationary phases, but the same mobile phase (methanol/0.01 M phosphate buffer, pH 5.8), that the introduction of glycine and taurine groups into the conjugated bile acids resulted in a constant selectivity between glycine and taurine acids, irrespective of the nature of the primary and secondary bile acids to which they were bonded. Therefore, it becomes obvious that the retention behavior of the glycine and taurine bile acids in RP-HPLC is generally controlled by the conjugated glycine and taurine groups. The conjugation selectivity can thus be used as an aid in the identification of the peaks of the glycine or taurine conjugates in RP-HPLC (60).

It may be done, for example, by calculating the selectivity factor of the first pair of conjugates in the chromatogram, tauroursodeoxycholic acid (TUDA) and glycoursodeoxycholic acid (GUDA). Then, knowledge of the retention times of the taurine conjugates can accurately predict the retention times of the glycine conjugates.

Solid Phase Extraction.

Solid phase extraction (SPE) is a sample preparation technique that utilizes disposable extraction columns (cartridges) that contain materials to extract, rapidly and quantitatively, specific compounds of interest from complex samples. The same principles of analyte/sorbent interactions that are exploited in the separation technique of HPLC are used in SPE. The extraction cartridges are packed with a variety of surface-modified bonded silica sorbents, which selectively retain specific classes of chemical compounds from within a given matrix. Bonded silica sorbents are in many ways the ideal materials for such isolation, primarily due to the number of different functional groups that can be readily bonded to the silica surface (65).

Bonded silica materials fall into four major categories based on the type of chemical interaction between the compound of interest and the bonded silica materials: non-polar, polar, ion exchange and covalent.(63, 64). A number of references describe method developments that help to select one of the extraction technique based on the chemical characteristics of the compound of interest. (61-69)

The process of extraction consists of four basic steps:

1. Conditioning. Preparing the cartridge for reproducible interaction with sample matrix by solvating the sorbent bed. This is done by passing a volume of an appropriate solvent through the cartridge, followed by a volume of a liquid similar in nature to the sample matrix. A common example of cartridge conditioning would be to pass methanol, followed by water, through a C 18 cartridge prior to extraction of an aqueous sample matrix.

2. Retention. Applying the sample to the conditioned cartridge results in the analyte, and perhaps other matrix components being retained on the sorbent surface due to one or more specific chemical interactions (e. g. Van der Waals or non-polar interactions between the hydrocarbon chain of an analyte and the hydrocarbon chain of a C-18 bonded phase). It should be pointed out that matrix contaminates may pass through the cartridge unretained, hence cleaning up the sample even at the retention or loading step.

3. Rinsing. Passing solvents through the cartridge rinses away additional interfering compounds while leaving the analyte undisturbed within the sorbent bed. A common rinse solvent for a non-polar extraction on C 18 sorbent would be water.

4. Elution. Passing an appropriate solvent through the cartridge which is specifically chosen to disrupt the analyte-sorbent interaction, resulting in selective elution of the analyte. For example, an organic solvent such as methanol would be a sufficiently strong solvent to disrupt the interaction between most non-polar analyses and C-18 bonded phase.(65)

SPE is used to prepare samples for analysis by methods such as HPLC, GC, NMR, RIA and TLC. It provides a clean, concentrated extract, which simplifies subsequent analysis by removing interfering components. It is used in a wide variety of applications for the identification and quantization of a number of endogenous compounds, including biologically active peptides, steroids, lipids, and catecholamines.

The technique is very powerful and useful in medical, food and beverage analysis, drug testing, and the evaluations of pollutants in soil and water. The extractions may take as little 5 minutes from start to finish and may lead to 90% recovery of the compound of interest from the sample matrix.

Bonded silica columns can be used with any solvent and with either strongly acidic or basic solutions(63). Some of the modifications of the technique utilize vacuum blocks, which help to extract 24 or more samples simultaneously and thus lend even more rapidity to an already rapid technique (64). All these advantages make this technique an irreplaceable part of a number of analytical methods.

CHAPTER TWO

HISTORICAL

Bile acid analysis using HPLC:

The separation of bile acids using HPLC was first proposed by Shaw and Elliott in 1976 (4). Using a Waters Corasil II column and a recycling mode,they found that it was possible to separate all of the common bile acid conjugates. However, in the analysis of human bile the method was found to be nonspecific. Later methods used a normal phase Perisorb A with gradient elusion (5) or a reversed phase μ-Bondapak C-18 column (6), but difficulty in resolving CDCA and DCA conjugates persisted even under these conditions.

Several other methods using μ-Bondapak C-18 column (7-11) or a Fatty Acid Analysis column (4-12) have successfully separated the dihydroxy conjugates in a mixture of conjugated bile acids. The 5α/5β epimers may be resolved using a normal phase system such as Corasil II in a recycling mode with a mobile phase consisting of acetonitrile/acetic acid 40:1 (v/ v) (9). An anion exchange packing, TSK Gel 1 EX 540 DEAE, has also been used in the group separation of the glycine and taurine conjugates, thereby providing a rapid method for the measurement of the glycine/taurine ratio in bile (13) .

One method of simplifying the analysis of the very complex mixtures of bile acids that can be obtained from biological materials was to perform a preliminary fractionation of the bile acids into unconjugated (free) bile acids, glycine conjugated bile acids (glycoconjugates), and taurine conjugated bile acids (tauroconjugates) using methods such as thin layer chromatography (TLC) (14) or ion exchange chromatography (IEC)

(15). Once fractionated, each group may then be resolved into its individual components using HPLC (16).

Both free and conjugated bile acids have been quantified by collection of HPLC eluent fractions, followed by enzymatic analysis (5, 18, 40-43). However, this method has been found to consume considerable amounts of enzyme neded , which can be a formidable problem from an economic standpoint since the enzymes used tend to be expensive. This problem has been largely overcome, however, by the development of post-column immobilized enzyme reactors. In particular, for bile acid analysis using HPLC, immobilized 3α-hydroxysteroid dehydrogenase (3α-HSD) enzyme has been used for post-column flow analysis (19).

The initial 3α-HSD system that was described for such analysis did not allow for the separation of glycodeoxycholic and taurochenodeoxycholic acids. It has been suggested as well, that the linear gradient elution in the HPLC system that was used for the separation may have caused problems in the reproducibility and the reliability of the enzymatic reaction.

Separation of the bile acids into fractions (free, glycoconjugates, and tauroconjugates) using PHP Sephadex LH 20 prior to HPLC has been recommended to alleviate the need for gradient elution (20). Improvements in enzyme immobilization, and in the selection of adequate mobile phase components, have led to enhanced separation and sensitivity in low level bile acid analysis (21, 22).

The resolution of the 3-sulfate conjugates of the common free and glycine and taurine conjugated bile acids using HPLC has been reported on a reversed-phase octadecylsilane (ODS) SC-02 column (23, 24). The application of this modified technique has been done by Rossi and co-workers (25), using an Altex Ultrex C-18 column for detection of sulfated and unsulfated forms of GLCA and TLCA, as well as the eight other minor conjugated bile acids in human bile.

Although there were many attempts to improve ultraviolet or fluorimetric detection via sophisticated derivatization techniques, many of which will be described in the next part of this historical review, there were some other ways to optimize the analytical procedure of simultaneous determination of conjugated bile acids in human fluids. Chi-Pui Pang and co workers (3) demonstrated a rapid way to separate ten conjugated bile acids using an isocratic mode and a C-18 column at ambient temperature. The one-step sample purification using C-18 solid phase extraction (Sep-Pak, C-18 cartridges) was used. This particular method, with modification, as described in chapters IV and IV, was the basis for the present work.

Another successful attempt was reported by Scalia, Pazzi et al (27). The injection loop was replaced with a precolumn that was dry packed with C-18 sorbent material (BB-C-18 cartridges). The diluted sample was directly injected into the chromatographic system. This modification allowed the analysis of practically any human fluid that contained conjugated bile acids (e.g., bile , urine , gastric juice, and serum) using regular reversed phase HPLC. The 10 common conjugated bile acids have been resolved with this method in all of the above fluids.

The very interesting and useful approach to reversed phase HPLC separation was made by Bourgulgnon and co-workers in 1994 (28). This method of optimization in irregularly shaped regions where the separation is dependent upon factors such as pH, solvent strength, or temperature was universal for reversed phase mode and could be used for the determination of the best conditional parameters for bile acid analysis. The universality of the method was due to mathematical algorithms that could be applied to find the feasible regions in which experimental optimization could be carried out. The biggest disadvantage of the method was the expense of the software (L.C. Resources Inc.) that was absolutely necessary for the success of the experiment.

The Most Popular Detection Methods in HPLC Bile acid Analysis:

HPLC has been widely used in bile acid analysis in many matrices. Its main advantage over other chromatographic procedures is its simplicity, since some classes of bile acids (glycine and taurine conjugates, for example) can be directly analyzed using conventional UV detectors at 193-210 nm without the need to perform preliminary derivatizations (3, 4, 9, 25, 26). However, bile acids do not have a strong UV absorbance and cannot be detected at 254 or 366 nm (useful filters in inexpensive UV detectors), so they have been detected and determined largely using differential refractometers (4, 15, 29).

The poor sensitivity of these devices, especially at very low concentrations of bile acids in some biological fluids, has prompted a search for alternative methods. Pre- and post-column derivatization procedures have been proposed.

Some derivatization reagents, such as 1-p nitrobenzyl-3-p-tolyltriazene (6), p-bromophenacyl-bromide (30), p- nitrophenyl esters (6, 31) and pentachlorophenyl esters (32), have been used to react with free and glycine conjugated bile acids before chromatographic separation to improve the sensitivity of UV detection . Although these derivatives show strong UV absorbance at 254 nm and methods are useful for the assay of bile acids in bile, the sensitivities are still insufficient to determine the small amount of bile acids present in human serum samples. In addition, since these reagents react only with carboxyl groups, taurine conjugates can not be determined using this method.

It has been reported that fluorescence derivatization methods may have the required sensitivity for low level bile acid analysis (33-35). Okuyama and co-workers (33) used preliminary esterification of free bile acids and glycine conjugates with 4 - bromomethyl-7-methoxylcoumarin followed by HPLC separation and detection with a fluorescence detector . The detection limit for individual bile acids was about 20 - 28 pmoles.

Kamada and co-workers (34) showed the determination of free and conjugated bile acids after the fractionation of bile and serum samples. All fractions were derivatized with 1-bromoacetylpyrene, a carboxylic acid derivatizing agent. The taurine conjugates were hydrolyzed using the bile acid hydrolytic enzyme cholylglycine hydrolase prior to derivatization to allow them to react. Derivatized bile acids were separated on a Redial- Pak A column and monitored using a fluorometer for detection. It was reported that detection limits for glycine and taurine conjugates were 5 pmoles and 10 pmoles, respectively. Ikava and co-workers (35), used a similar technique for the determination of bile acids in human feces.

Shimada et al. (44, 50) used inclusion chromatography with cyclodextrin (CD) as a mobile phase additive in HPLC of bile acids pre-labeled with the same reagent (1-bromoacylpyrene). It was found that inclusion chromatography was more appropriate for separation of derivatized bile acids than conventional chromatography. Guldutuna and co-workers applied a similar method for the determination of free and conjugated bile acids that were pre-labeled with 4-bromomethyl-7-methoxycoumarin. Taurine conjugates were hydrolyzed prior to the derivatization with the same reagent (34). The analysis of bile acids in serum , liver biopsies, bile, gastric juice and feces were performed(36). The most recent application of this technique has been reported by Cavrini and co-workers (49) using of 2-bromoacetyl-6-methoxynaphthalene for the determination of ursodeoxycholic acid and chenodeoxycholic acid.

The free bile acids may also be analyzed by HPLC using an ion pair reagent, such as Hyamine 1622, with UV monitoring of the ion pair at 254 nm (17). Although the dihydroxy bile acids were not satisfactorily separated using this method, the detection limit was 0.5 pmole. Using modified ion pair separation of conjugated bile acids in human bile has been performed by Wildgrube and co-workers (37). Quantization was done using UV absorption at 214 nm. Later, using a similar method, they determined the biliary pattern of conjugated bile acids in human, dog, and rabbit (38). Recently this

method was applied to the analysis of pig bile by Legrand-Defretin and co-workers. The method was found to be simple and efficient because it could be performed without prior treatment of the samples (39).

As mentioned earlier, the use of the immobilized enzyme 3α-hydroxysteroid dehydrogenase (3α-HSD) as a post-column reactor along with a reactant solution of the dehydrogenase enzyme co-factor NAD^+ and fluorimetric detection became a very popular method that has been modified and developed by many researchers (19, 21, 22, 40-48). In these systems, the bile acids in eluates from analytical columns were reacted with NAD^+ in the 3α-HSD enzyme post-column reactors to produce NADH, which was monitored using a fluorometer. The detection limits were reported as 0.13 - 0.28 pmole. This technique was applied with chemiluminescence detection for the determination of glucose, bile acids, and ATP (51). Tazawa and co-workers determined urinary bile acids of non-cholestatic and cholestatic infants using two detection systems: UV detection for the determination of the non-3α-hydroxy bile acids, and fluorescence detection for the determination of the 3α-hydroxy bile acids (52).

The use of electrochemical detection of NADH (53) rather then fluorimetric detection does not appear to enhance the sensitivity of the method, although its specificity may be increased. The main draw back of methods using pre- and post-column derivatization procedures is that they are time consuming and additional steps are necessary that render the procedure not easily controllable .

A recent method used to detect bile acids previously separated by HPLC has been evaporative light scattering. Roda et al. (16) compared two methods of detection: UV detection at 200 nm and detection with an evaporative light scattering mass detector (ELSD II). The detection limit of the ELSD II was in the order of 2-7 nmole for either free or amidated bile acids, while with UV detection the detection limit of glycine or taurine conjugated bile acids was 4-6 nmole and for free bile acids it was reported as 220-280 nmole. ELSD II was thus much better for the analysis of the non-conjugated bile

acids. The usefulness of this technique is especially seen in the analysis of non-fractionated bile samples.

The comparison of the above reported methods indicates that all of them, in some degree, can be useful for a particular assay of bile acids, mostly depending upon what kind of study is to be performed and what type of bile acid sample will be analyzed (26). The detection of bile acids following HPLC separation is an area of analysis that merits further investigation.

CHAPTER THREE

STATEMENT OF PROBLEM

Separation and analysis of bile acids using HPLC remains a difficult and challenging task. This observation is supported by the large number of analytical methods published each year that use various types of pre- and post-column reaction schemes with various types of detectors. Many of the advantages and disadvantages of some of the available methods have been discussed in the historical review presented in Chapter 2, but most of the referred methods bear a lack of simplicity and, as a consequence, are very time consuming. It is for this reason that those methods that provide quick and relatively simple ways to determine bile acids in biological matrices deserve special attention for their modification and development.

One such method was used as an initial model for the study described in this manuscript (3). The primary purpose of this research was the modification and validation of the method using different chromatographic equipment. To accomplish this, it was necessary to optimize work parameters such as pH, mobile phase composition, and flow rate for the developed method. Such drastic modifications generally also require validation of the method to be performed.

The analytical method, once modified and validated, was then to be used for analysis of patient bile samples obtained from St. Elizabeth's Hospital Medical Center, Youngstown, Ohio. The method was also intended for full development so that it could serve as a comparison for a separately developed bile analysis method using immobilized cholylglycine hydrolase and HPLC.

CHAPTER FOUR

MATERIALS AND METHODS

Reagents

Methanol (HPLC grade) and potassium dihydrogen phosphate were obtained from Fisher Scientific Co. (Fair Lawn , NJ). Tauroursodeoxycholic acid (TUDA), taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDA), taurodeoxycholic acid (TDCA), glycochenodeoxycholic acid (GCDA), glycodeoxycholic acid (GDCA), taurolithocholic acid (TLCA), glycolithocholic acid (GLCA), and 5β-pregnane-3α,17α-diol-20-one were obtained from Sigma Chemical Co. (St. Louis, MO). Glycoursodeoxycholic acid (GUDA) were from Calbiochem Chemical Co. (La Jolla, CA), and Maxi-Clean™ C-18 SPE (solid phase extraction) cartridges from Alltech Associates Inc. (Deerfield, IL).

Apparatus

Chromatographic separations and analysis were carried out using a 250 mm x 4.6 mm I.D. Adsorbosphere, C-18, 5 μm particle size reversed phase column purchased from Alltech Associates, Inc. (Deerfield , IL), as were all connectors and tubing. Model 110B Pumps (Beckman Instruments, Fullerton, CA) were used to deliver the mobile phase. A Model 338 analog interface (Beckman Instruments) was used to interface the chromatography instrumentation with the Beckman System Gold chromatography program (System Gold software Version 5.0). For detection of analytes, a Beckman Instruments Model 166 Programmable Variable Wavelength UV/Visible detector was used, with detection at 215 nm. The system configuration can be seen in Figure 2.

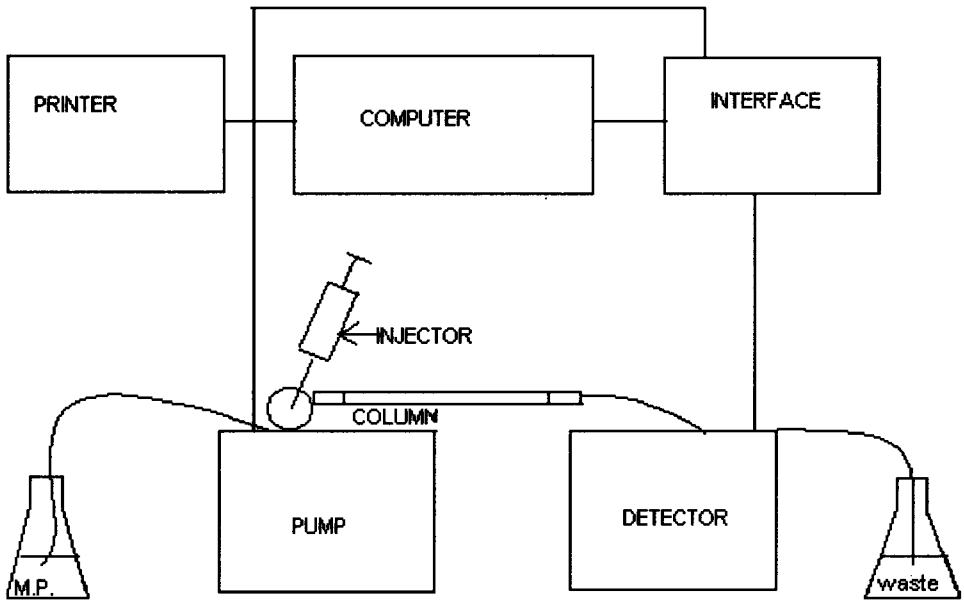


Fig. 2 SYSTEM CONFIGURATION

Bile collection

Blind samples were obtained from Dr. Vincent Vanek at St. Elizabeth Hospital, where they were collected from patients suffering from cholecystitis (from the gall bladder during cholecystomy). Control samples were also obtained from cadavers by autopsy. The specimens were collected in polystyrene bottles and stored at -70.0°C.

Sample treatment

An aliquot (0.5 mL) of a bile sample, after thawing, mixing, and centrifugation (3000 x g, 10 min.) was added to 25 mL of 30 mM potassium dihydrogen phosphate buffer, pH 6.25. To a 5.00 mL portion, 10 mL of deionized water and 50 μL of 5.98 mM 5β-pregnane-3α,17α-diol-20-one dissolved in methanol was added as an internal standard. The diluted bile sample was then applied to a Maxi-Clean™ C-18 SPE cartridge, which had been pre-treated with 5 mL methanol and 30 mL water in a Maxi-Clean™ cartridge rack (Alltech Associates, Deerfield, IL). After application of the bile sample, the cartridge was washed in turn with 10 mL of water, 3 mL of 10% acetone (v/v), and 10 mL of water . The bile acids were then eluted from the cartridge using 3 mL of methanol . The eluate was evaporated to dryness in a stream of nitrogen at 37°C. The sample was reconstituted by sonication in 0.550 mL of mobile phase prior to injection (20 μL) into chromatographic system.

Chromatography

An isocratic mobile phase consisting of 65% (v/v) methanol in 0.050 M potassium dihydrogen phosphate, pH 4.10 was used for the chromatographic separation. The aqueous and organic components of the mobile phase were filtered through a 0.45 μ m Nylon-66 membrane (Alltech Associates, Inc., Deerfield , IL). A reversed phase C-18 column was used at ambient temperature. The eluate was monitored at 215 nm with a

flow rate of 1.5 mL/min. The total run time was 45 min., after which the system was immediately ready for another injection.

CHAPTER FIVE

RESULTS AND DISCUSSION

Recovery study

Bile is a complicated mixture of waste products, many of which are very non-polar (bile pigments, for example) and will stick nearly irreversibly to chromatographic columns and degrade their performance. In addition to the non-polar interferences present in bile, there are also present many other substances that efficiently absorb UV light and thus complicate the chromatographic record, making it difficult to interpret. Thus, in order to preserve the quality of the HPLC column in this analysis and to simplify the chromatogram to make identification and quantitation easier, it was necessary to perform an isolation and purification of the bile samples before the chromatographic separation of the bile acids. This clean-up step required the use of solid phase extraction tubes, small plastic devices containing octadecyl chromatographic packing material that selectively retained the bile acids.

Since the bile samples were run through the Maxi-Clean™ C-18 SPE cartridges during the isolation procedure, and since several manipulations were done on the sample after the extraction, it was necessary to have some idea of how much sample was lost (if any) in the clean-up procedure. In order to determine the magnitude of this, sample recovery was determined.

The following three chromatographic determinations were performed in duplicate for each sample that was used for the recovery study.

1. A chromatogram of the middle standard concentration (6.92 mg/mL), with 50 μL of internal standard added to 0.500 mL of the standard.

2. A chromatogram of a mixture of 0.250 mL of bile and 0.250 mL of the middle concentration standard (i.e., the same as used in 1) with an addition of 50 μL of internal standard. The mixture was diluted with 25 mL of phosphate buffer (30 mM) and then run through the clean-up procedure before the injection.

3. A chromatogram of the bile sample (i.e., the same as used in 2) that was prepared from 0.500 mL of bile with 50 μL of internal standard added, diluted with 25 mL of phosphate buffer and run through the clean-up procedure.

For each duplicate determination, the peak heights of the individual bile acids and the internal standard were averaged and used for calculation. For each bile acid on the chromatograms, the actual recovery was determined directly from the peak heights and the corrected recovery was determined from the internal standard ratio (IS ratio). The IS ratio was calculated as the ratio of each bile acid peak height divided by the internal standard peak height from the same chromatogram.

To calculate the actual recovery of the individual bile acids, the value of the peak height for the individual bile acids in the chromatogram of the mixture (sample 2, above) was compared to the expected peak height. The expected peak height for sample 2 was calculated by dividing the peak heights of the individual sample (sample 1, above) and standard (sample 3, above) by two and adding them together. The percent recovery was determined as:

$$\% \text{ Recovery} = \frac{actual\ peak\ height}{expected\ peak\ height} * 100\%$$

To calculate the corrected recovery of the individual bile acids, the value of the IS ratio for the mixture of bile and standard, sample number 2 as outlined above, was used without change, but the values of the IS ratios both of the bile sample and the standard

was divided in half and then added together. That was because in the mixture of bile and standard, half amounts of bile and standard were used. Then the recovery was calculated by the comparing the IS ratio of the mixture and the sum of the bile and standard half IS ratios, as outlined below.

$$\% \text{ Recovery} = \frac{\textit{Actual IS Ratio of Std. \& Bile Mixture}}{\textit{Expected IS Ratio of Std. \& Bile Mixture}} * 100\%$$

where,

$$\textit{Actual IS Ratio} = \frac{\textit{Actual Peak Height}}{\textit{Peak Height of Internal Standard}}$$

and,

$$\textit{Expected IS Ratio} = \frac{\left[\textit{Actual Peak Height of Standard}/2 + \textit{Actual Peak Height of Bile}/2\right]}{\textit{Peak Height of Internal Standard}}$$

The purpose of the addition of the IS to each analytical sample was to diminish errors due to handling of the sample. Since each sample contained exactly the same amount of IS it was possible to correlate the loss of IS with the loss of bile acids during the isolation procedure.

Three bile samples were used for the recovery study and the data are shown in Tables 2 and 2a. From the data, several observations can be made. In looking at the uncorrected recoveries, it was apparent that a significant amount of loss was occurring due to sample handling. However, the corrected recoveries showed that using the internal standard tended to overcorrect the recoveries. This apparent disparity in the data can be explained by the different behavior of the internal standard due to its differing chemical structure.

As can be seen in Figure 3, the conjugated bile acids that were of interest in this study were highly polar compounds, primarily due to the presence of glycine or taurine

taurine conjugates in addition to the -OH groups on the phenanthrene ring structure. The conjugated glycine and taurine were not present in the internal standards structure. Thus, even though the steroid nucleus of the internal standard and of any of bile acids were practically the same, the differences in the polarity due to the conjugated amino acids would be expected to cause a slight variation in the way these compounds were absorbed during the isolation procedure. As such, and since the variation should be consistent from sample to sample, the recovery data was used as a correction factor in the final calculation.

Table 2

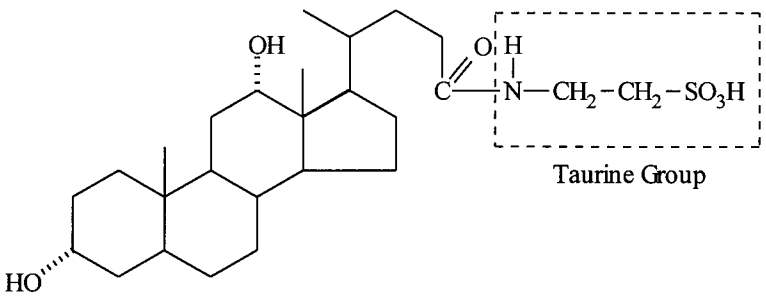
Uncorrected Recovery of Bile Acids
No Internal Standard

Bile Acid	Average Recovery (%)
TUDA	37.3
GUDA	39.4
TCA	57.8
GCA	62.8
TCDA	54.6
TDCA	52.6
GCDA	62.5
GDCA	61.3
TLCA	38.6
GLCA	29.7

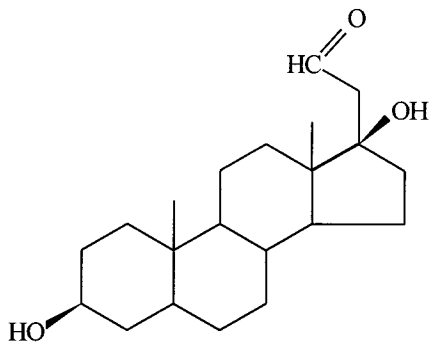
Table 2a

Corrected Recovery of Bile Acids
Internal Standard

Bile Acid	Average Recovery (%)
TUDA	95.0
GUDA	106.9
TCA	118.8
GCA	107.4
TCDA	116.7
TDCA	121.1
GCDA	109.2
GDCA	107.1
TLCA	132.5
GLCA	95.6



a) Taurodeoxycholic acid
Taurine group provides additional polarity.



b) 5-β pregnane-3α, 17α-diol-20-one
(Internal Standard)

Fig. 3. Structures of a) conjugated bile acid and b) the Internal Standard

Bile acid analysis

Satisfactory separation and detection of the 10 major conjugate bile acids present in human bile was obtained. As seen in Figure 4, a standard mixture of the 10 major bile acids was well separated. The analytical system was relatively simple since there was no gradient elution involved and therefore no gradient mixer or controller was required. The use of an isocratic mobile phase with a constant flow rate was satisfactory for the analysis of bile samples (Figure 5). This method differed from other methanol-phosphate buffer systems that have been used for analysis of bile acids by using a more acidic pH. Indeed, exact control of the buffer pH was essential for the high resolution that was achieved in a relatively short period of time (45 min.).

This excellent resolution was obtained by adjustment of the mobile phase composition with a relatively (to other methods) small concentration of organic component (65%). The aqueous phosphate buffer concentration of 0.050 M was found to be critical for complete resolution of the bile acids and the internal standard, which was an absolutely necessary component of the analysis. The relative increase of the aqueous component of mobile phase to 35% phosphate buffer resulted in an increased retention of all of the bile acids over that which had previously been reported, but it made the resolution of all the 11 components of the standard considerably more pronounced.

Exact overlap of the corresponding bile acids peaks was obtained when a bile sample was spiked with authentic bile acid standards prior to sample treatment (figure 6). The chromatographic analysis required about 45 minutes. No guard column was used to avoid peak broadening.

The one-step sample purification with the Maxi-Clean™ C-18 cartridges was able to replace solvent extraction or XAD-2 ion-exchange treatment of the bile specimens (54). The solid phase extraction method was found to be quite adequate for sample clean-up and gave satisfactory recovery of the bile acids (see the section on Recovery Study,

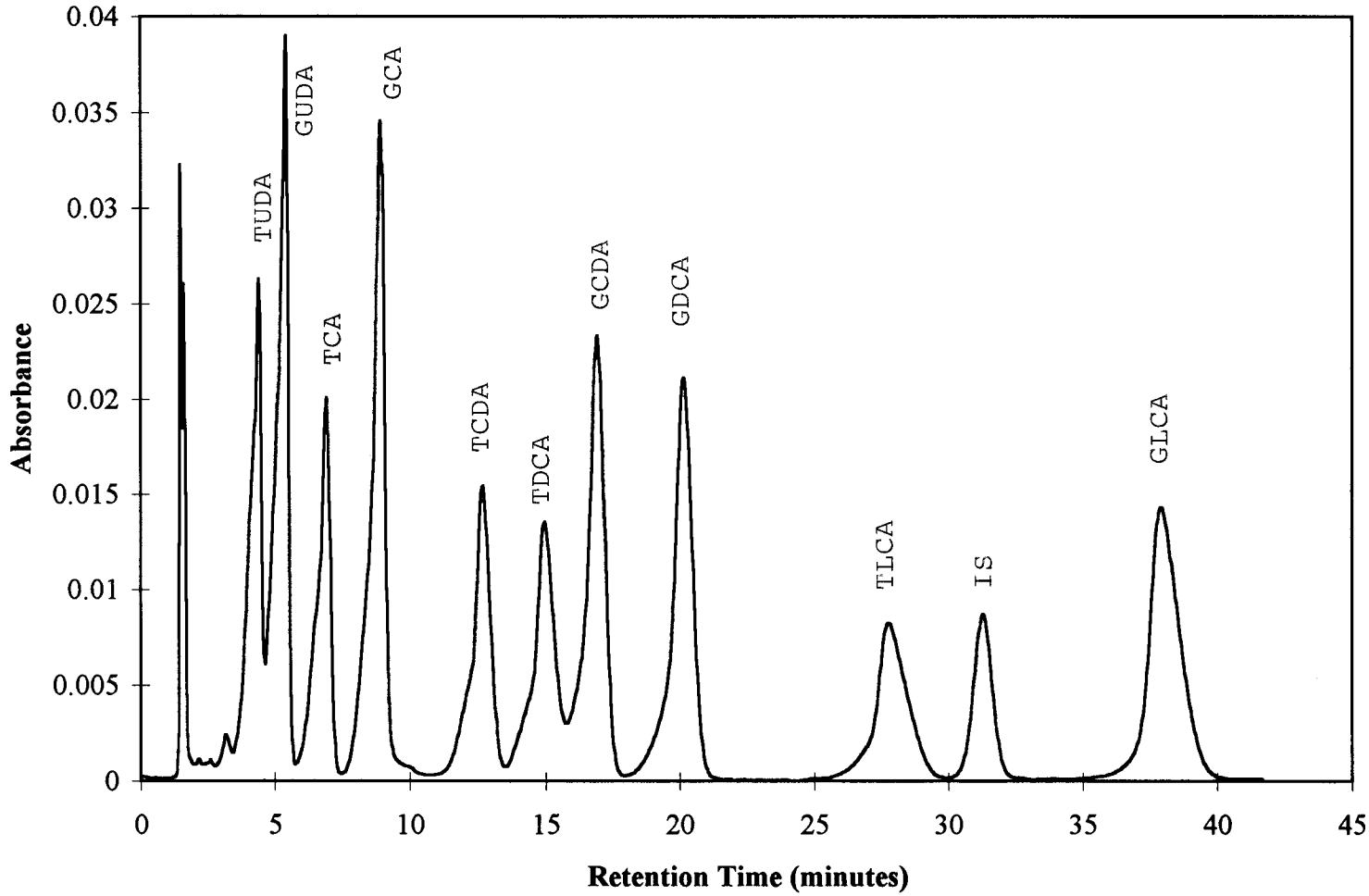


Fig.4 Chromatogram of Standard Bile Acid Mixture.

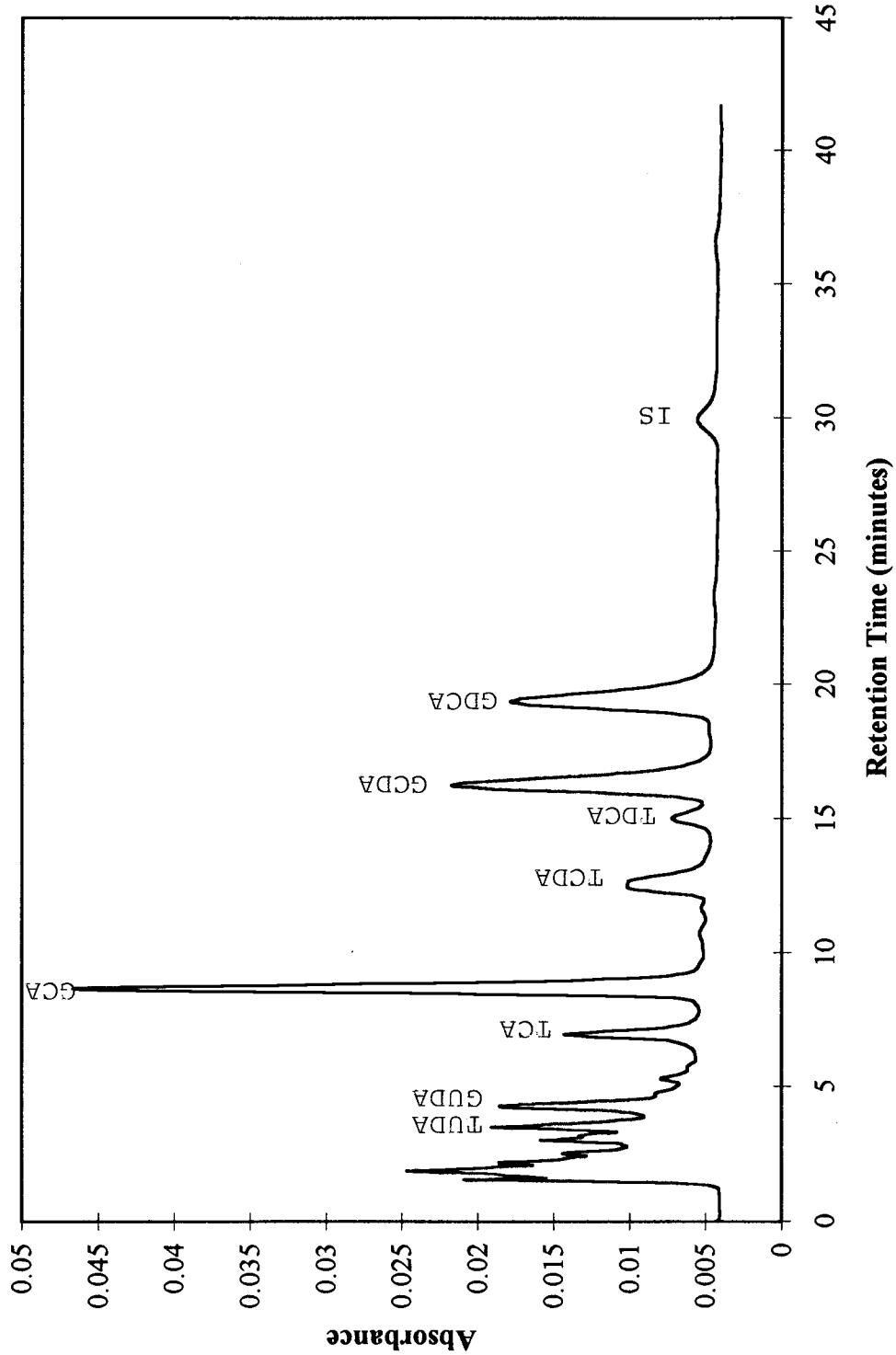


Fig.5 Chromatogram of Bile Sample.

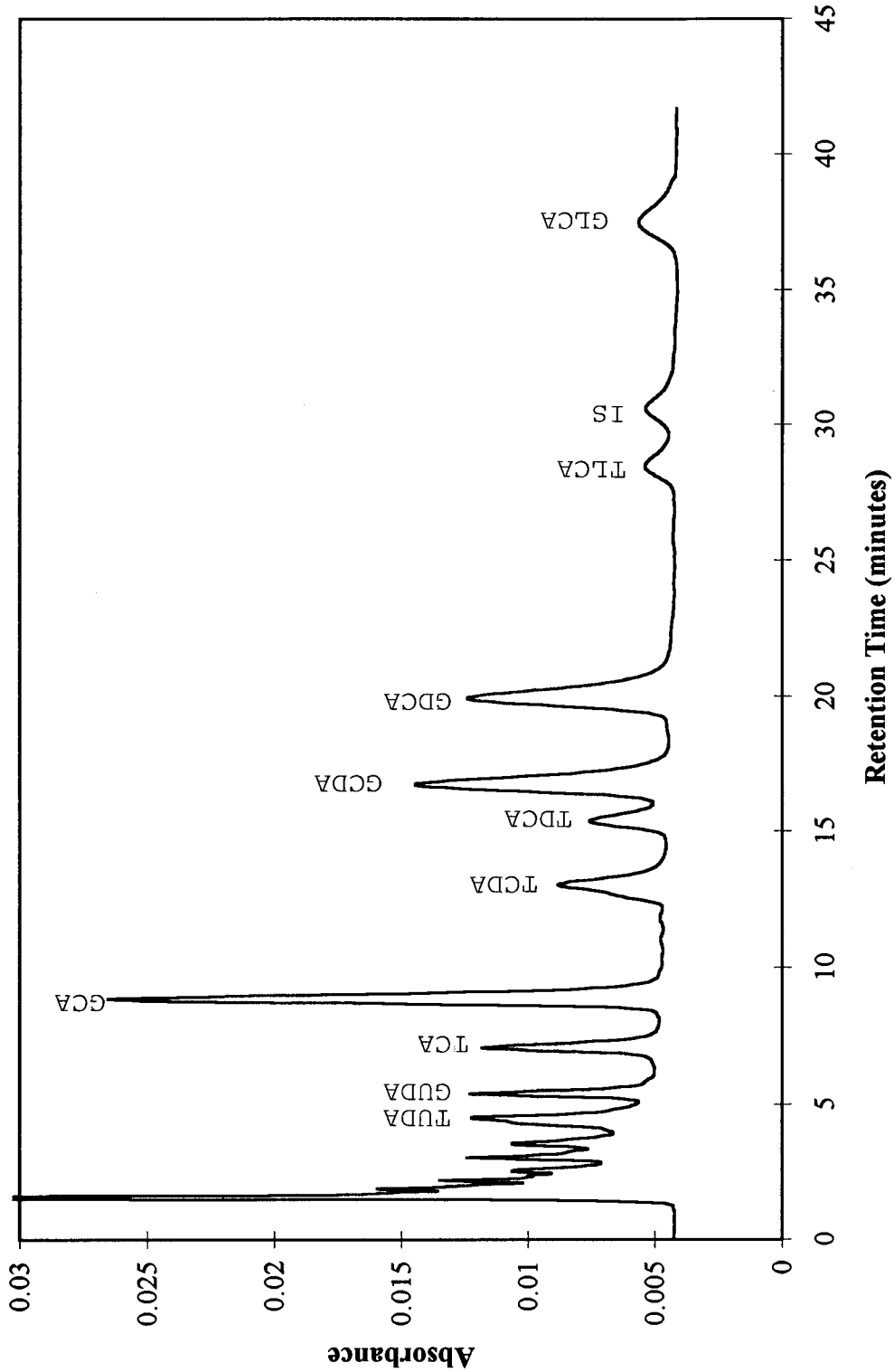


Fig.6 Chromatogram of Bile Acid Mixture Spiked with Bile Sample.

above). The suitability of Sep-Pak C-18 SPE cartridges (Waters Associates, Milford, MA), which have been widely used for bile acid isolation, and Maxi-Clean™ C-18 SPE cartridges (Alltech Associates, Deerfield , IL) were tested and compared. After several extractions were done and the results compared, the Maxi-Clean™ C-18 cartridges proved easier to use and appeared to give better and more consistent performance. The Maxi-Clean™ C-18 cartridges were therefore used for the solid phase extractions in all of bile analyses that were subject of this report.

Data for the analysis of bile samples were obtained by comparison of the analysis of a mixture of the 10 conjugated bile acid standards (6.92 mg/mL) to individual bile samples prepared the same day. The analytical data are presented in Table 3. All data presented in this report were obtained by peak height measurement. Two correction factors were used in the calculation and the calculation is shown below.

$$C_{BA,\,Sample} = \frac{\left(\frac{P.H._{BA,\,Sample} / P.H._{IS,\,Sample}}{P.H._{BA,\,Std.} / P.H._{IS,\,Std.}} \right) * C_{BA,\,Std.} * 5}{R_{BA}}$$

Where,

$C_{BA,\,Sample}$ = concentration of bile acid in the sample

$C_{BA,\,Std.}$ = concentration of bile acid in the standard

$P.H._{BA,\,Sample}$ = peak height of bile acid in the sample

$P.H._{BA,\,Std.}$ = peak height of bile acid in the standard

$P.H._{IS,\,Sample}$ = peak height of internal standard in the sample

$P.H._{IS,\,Std.}$ = peak height of internal standard in the standard.

R_{BA} = correction factor determined from % recovery of the individual bile acid

$$R_{BA} = \% \; Recovery \; of \; Bile \; Acid / 100$$

The correction factor, 5, is a dilution factor since 5 mL of a 25 mL aliquot was taken for analysis

The internal standard played an extremely important role in both the sample and standard runs, since its concentration was known in every chromatogram (0.1 mg dissolved in 50 μL of methanol). At the same time, it's presence as the same mathematical component in the numerator and denominator as a part of IS ratio cannot change the general ratio of the bile acids since it is self-canceling.

The results of the calculations assembled in Table 4 were provided to St. Elizabeth Hospital Medical Center for further medical interpretation. In general, the results show that the concentrations of conjugated bile acids in bile of patients with non-biliary disease (control group) were much higher than those of patients with gallstones (Table 3). The same correlation was found in the assay of Pang and co-workers (3) whose method was modified and described in the present work, and whose results are summarized in Table 3a, below.

Table 3

Concentrations (mg/mL) of Bile Acids in Gall Bladder Bile
of Control and Study Patients

CONTROL PATIENTS (n = 15)

Bile Acid	Mean	Std. Dev.	Range
TUDA	9.72	6.30	0.82 - 21.7
GUDA	7.57	6.54	1.51 - 21.1
TCA	19.05	16.77	1.67 - 56.8
GCA	28.96	25.97	3.64 - 94.0
TCDA	19.66	20.05	2.50 - 62.6
TDCA	11.19	13.85	3.12 - 55.5
GCDA	44.60	38.52	9.78 - 144.0
GDCA	43.30	31.03	10.27 - 111.0
TLCA	0.70	0.81	0.00 - 2.25
GLCA	1.19	0.96	0.00 - 3.47

STUDY PATIENTS (n = 11)

Bile Acid	Mean	Std. Dev.	Range
TUDA	1.84	1.90	0.02 - 6.52
GUDA	5.54	6.07	0.04 - 17.8
TCA	7.12	5.12	0.18 - 16.1
GCA	11.43	8.63	0.89 - 25.0
TCDA	7.65	6.26	0.25 - 18.7
TDCA	5.05	3.72	0.11 - 11.0
GCDA	19.73	18.47	0.80 - 59.0
GDCA	16.33	14.02	0.28 - 49.2
TLCA	0.52	0.55	0.00 - 1.93
GLCA	1.24	1.20	0.00 - 4.38

Table 3a
Literature Concentrations of Conjugated Bile Acids in Human Bile (reference)

CONTROL PATIENTS (n = 20)

Bile Acid	Mean	Std. Dev.	Range
TUDA	0.94	0.94	0 - 3.6
GUDA	4.53	3.58	0.57 - 13.77
TCA	7.31	5.43	1.29 - 18.66
GCA	17.09	9.68	3.59 - 40.74
TCDA	11.79	7.2	3.12 - 26.66
TDCA	2.4	4.8	0 - 21.7
GCDA	31.17	14	6.08 - 49.05
GDCA	6.65	8.63	0 - 35.84
TLCA	0.22	0.31	0 - 1.11
GLCA	0.43	0.74	0 - 3.38

STUDY PATIENTS (n = 25)

Bile Acid	Mean	Std. Dev.	Range
TUDA	0.68	0.99	0 - 4.85
GUDA	1.7	2.17	0 - 10.75
TCA	5.16	3.93	0.48 - 13.55
GCA	11.36	6.19	2.05 - 23.00
TCDA	5.95	3.29	0.89 - 12.89
TDCA	3.34	6.99	0 - 34.8
GCDA	12.78	6.74	0.8 - 25.37
GDCA	6.89	4.72	0 - 15.75
TLCA	0.04	0.09	0 - 0.38
GLCA	0.21	0.28	0 - 1.04

Table 4

Concentrations (mg/mL) of Conjugated Bile Acids in Human Bile

Bile Acid	Std. Conc.	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 7
TUDA	1.412	2.02	2.28	1.49	0.05	1.59	1.77
GUDA	1.414	3.33	1.67	1.58	0.04	3.24	16.3
TCA	1.417	8.53	2.27	7.04	0.18	6.36	9.33
GCA	1.406	13.6	1.51	6.32	0.89	14.7	14.6
TCDA	1.412	15.4	1.70	5.47	0.25	6.17	7.86
TDCA	1.410	6.38	1.53	5.79	0.11	4.77	7.02
GCDA	1.426	39.0	1.51	13.0	0.80	25.7	17.7
GDCA	1.341	18.3	1.46	14.2	0.28	20.9	23.9
TLCA	1.418	0.38	0.81	0.86	0.00	0.36	0.33
GLCA	1.395	0.45	1.54	1.59	0.00	0.90	0.59

Table 4 (continued)

Bile Acid	Std. Conc.	Sample 8	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14
TUDA	1.412	0.60	3.61	14.14	14.82	7.72	9.15
GUDA	1.414	17.8	6.82	1.60	17.16	11.7	21.1
TCA	1.417	1.46	12.0	11.37	14.18	19.1	48.7
GCA	1.406	2.22	25.0	33.18	8.28	56.0	94.0
TCDA	1.412	1.14	18.7	8.46	26.50	21.6	49.6
TDCA	1.410	0.73	4.96	5.83	7.98	21.3	21.7
GCDA	1.426	1.72	59.0	25.6	24.44	105	144
GDCA	1.341	1.44	23.3	20.39	10.27	101	65.9
TLCA	1.418	0.25	0.64	0.44	1.68	1.61	2.16
GLCA	1.395	0.32	1.24	0.70	1.55	1.72	1.29

Bile Acid	Std. Conc.	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20
TUDA	1.412	5.52	7.99	5.25	6.08	18.5	1.43
GUDA	1.414	1.89	4.07	11.36	3.27	6.10	1.44
TCA	1.417	5.33	14.9	24.23	40.8	6.68	5.88
GCA	1.406	15.0	35.2	48.96	4.63	13.5	9.78
TCDA	1.412	3.76	11.7	22.98	2.50	6.81	7.64
TDCA	1.410	6.10	3.12	17.04	3.84	4.18	5.42
GCDA	1.426	21.7	41.2	65.26	9.78	20.9	19.5
GDCA	1.341	34.5	19.0	66.23	51.3	39.3	13.0
TLCA	1.418	0.24	0.50	0.85	0.21	0.40	0.00
GLCA	1.395	0.55	2.65	3.47	1.87	1.00	0.40

Bile Acid	Std. Conc.	Sample 21	Sample 22	Sample 23	Sample 24
TUDA	1.412	0.82	6.52	1.61	0.26
GUDA	1.414	1.51	4.16	5.64	0.33
TCA	1.417	1.67	12.3	16.1	2.74
GCA	1.406	7.95	19.1	22.0	4.83
TCDA	1.412	62.6	13.7	10.9	2.87
TDCA	1.410	3.93	11.0	10.9	2.39
GCDA	1.426	11.4	15.6	35.5	8.19
GDCA	1.341	43.5	17.2	49.2	9.43
TLCA	1.418	0.00	1.93	0.00	0.17
GLCA	1.395	0.00	4.38	1.98	0.66

Table 4 (continued)

Bile Acid	Std. Conc.	Sample 27	Sample 28	Sample 29	Sample 30
TUDA	1.412	5.24	21.7	2.62	3.12
GUDA	1.414	3.02	16.7	4.14	8.52
TCA	1.417	5.34	56.8	14.5	16.3
GCA	1.406	8.84	49.8	3.64	45.7
TCDA	1.412	3.66	54.0	7.78	5.33
TDCA	1.410	3.95	55.5	3.64	4.37
GCDA	1.426	18.6	79.3	33.9	48.4
GDCA	1.341	23.5	111	15.1	35.5
TLCA	1.418	0.22	2.25	0.00	0.00
GLCA	1.395	0.96	1.20	0.00	0.50

Method validation

In addition to the recovery studies outlined above, studies of long and short term reproducibility, linearity, and detection limits were performed, in order to further validate the method for analysis.

The detector was found to be linear with increasing concentration. A sample calibration curve is shown in Figure 7. In the figure, it can be seen that, while the calibrations for each bile acid do not overlap, they were all linear. The linearity data is summarized in Table 5.

It was not necessary to use any kind of chemical prelabeling or post-column derivatization to improve the sensitivity of detection for the conjugated bile acids. This was because it was found that the detector was sensitive enough for the analysis of that kind that is necessary for human bile. As known from other researchers, the maximal absorbance for amidated bile acids occurs around 200 nm, specifically 193-210 nm (3, 4, 9, 25). In our case the wavelength of the detector was slightly increased to 215 to avoid unwanted noise. The detection limits for the individual bile acids was in a range of 0.022 - 0.195 mM/L and is tabulated in Table 6.

Fig.7 **Conjugated Bile Acid Standard Curves Using UV Detection**

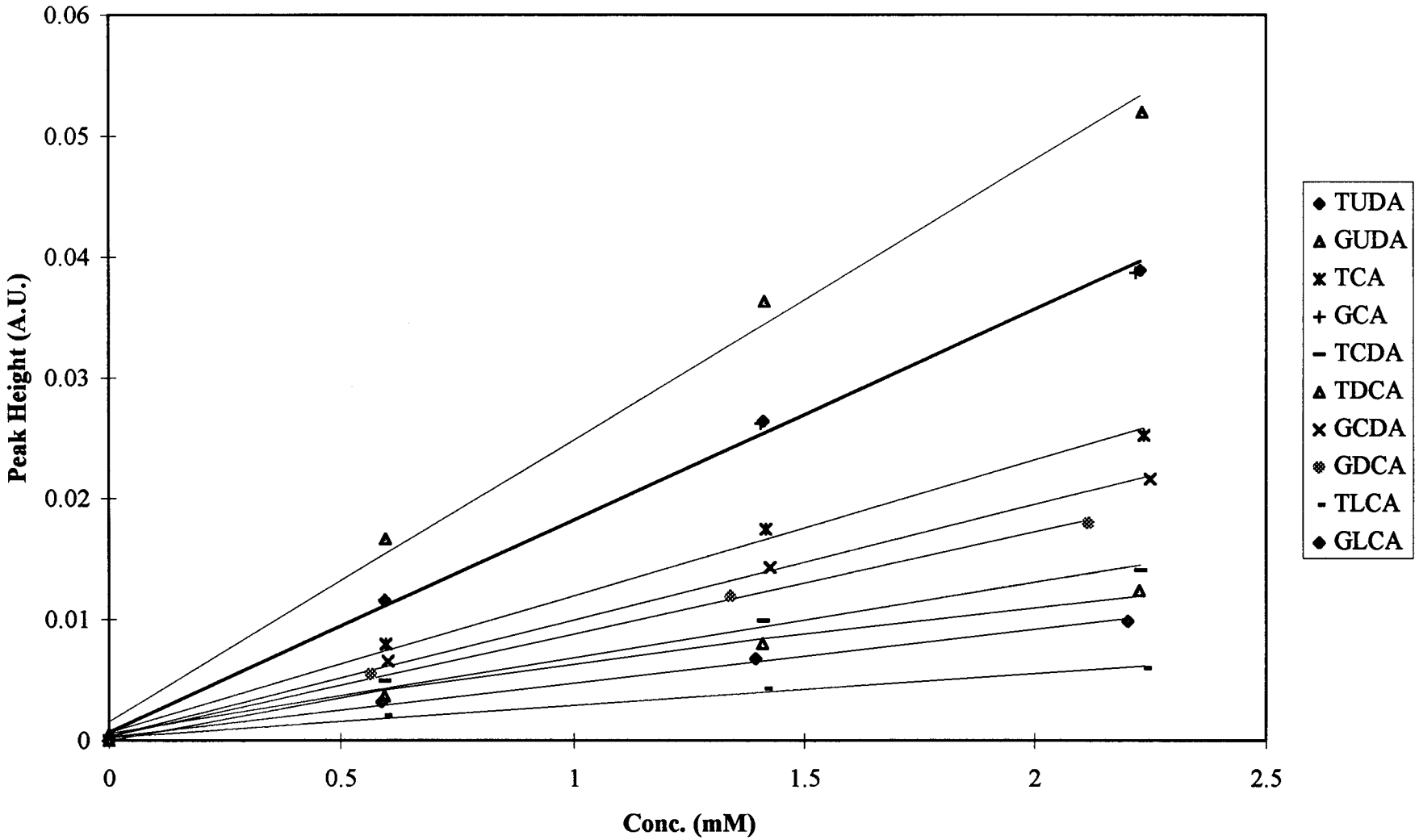


Table 5

Linearity Data for Conjugated Bile Acids

Bile Acid	Slope	Intercept	Corr. Coeff.
TUDA	0.01749	0.000692	0.999
GUDA	0.02325	0.001564	0.997
TCA	0.01126	0.000684	0.998
GCA	0.01744	0.000714	0.999
TCDA	0.00622	0.000616	0.995
TDCA	0.00531	0.000519	0.995
GCDA	0.00956	0.000387	0.999
GDCA	0.00843	0.000356	0.999
TLCA	0.00264	0.000268	0.994
GLCA	0.00445	0.000293	0.997

Table 6

Detection Limits for the Conjugated Bile Acids

BILE ACID	DETECTION LIMIT (mM)
TUDA	0.029
GUDA	0.022
TCA	0.046
GCA	0.030
TCDA	0.083
TDCA	0.097
GCDA	0.054
GDCA	0.072
TLCA	0.195
GLCA	0.116

Studies were also performed to establish the long and short term stability of the equipment and method in general. The studies were made: a) during a single day by measuring the bile acid peak heights in the standard mixture chromatograms (n = 3) and b) during the entire period of analysis, which covered 33 days, by comparing the standard

bile acid peak heights and retention time daily. The results of the long and the short term stability studies are shown in Tables 7 and 8.

It can be seen from the tables that the relative standard deviation (RSD) values indicated a very good reproducibility of analysis in both the short term and long term studies. For the single day study in Table 7, the retention values of the bile acids were practically invariant, with the RSD values staying around 1%. The range of values was 0.881% to 1.287%, with a mean RSD of 1.089%. The variability of the peak heights during a single day was also relatively low, staying well below 10%. The range of values was 2.357% to 9.171%, with a mean RSD of 6.970%.

For the long term study shown in Table 8, the values showed more variability, but were still quite good considering the length of the study. The most pronounced effect was an increased RSD of both the retention times and peak heights as the retention time increased. This was to be expected since the most likely cause of instability over this length of time was the flow rate of the mobile phase and condition of the column (i.e., normal degradation of the stationary phase with time). The range of values for the retention time RSD values was 2.570% to 5.595%, with a mean RSD of 4.159%. The range of RSD values for the peak heights was 10.211% to 25.367%, with a mean RSD of 16.724%.

Table 7

Short Term Stability for Retention Time and Peak Height

TUDA	Ret. Time (min)	Pk. Ht. (A.U.)		GUDA	Ret. Time (min)	Pk. Ht. (A.U.)
1	4.397	0.02287		1	5.436	0.03389
2	4.479	0.02559		2	5.512	0.03787
3	4.441	0.02566		3	5.423	0.03857
Mean	4.439	0.02471		Mean	5.457	0.03678
Std. Dev.	0.041	0.00159		Std. Dev.	0.048	0.00252
RSD (%)	0.924%	6.439%		RSD (%)	0.881%	6.864%

TCA	Ret. Time (min)	Pk. Ht. (A.U.)		GCA	Ret. Time (min)	Pk. Ht. (A.U.)
1	6.903	0.01739		1	8.958	0.02933
2	7.060	0.0197		2	9.107	0.0333
3	6.935	0.01966		3	8.937	0.03395
Mean	6.966	0.01892		Mean	9.001	0.03219
Std. Dev.	0.083	0.00132		Std. Dev.	0.093	0.00250
RSD (%)	1.191%	6.990%		RSD (%)	1.030%	7.768%

TCDA	Ret. Time (min)	Pk. Ht. (A.U.)		TDCA	Ret. Time (min)	Pk. Ht. (A.U.)
1	12.605	0.01313		1	14.889	0.01155
2	12.917	0.01504		2	15.244	0.01315
3	12.674	0.01515		3	14.959	0.01328
Mean	12.732	0.01444		Mean	15.031	0.01266
Std. Dev.	0.164	0.00114		Std. Dev.	0.188	0.00096
RSD (%)	1.287%	7.866%		RSD (%)	1.251%	7.610%

GCDA	Ret. Time (min)	Pk. Ht. (A.U.)		GDCA	Ret. Time (min)	Pk. Ht. (A.U.)
1	16.982	0.02004		1	20.242	0.02070
2	17.26	0.02251		2	20.570	0.01998
3	16.921	0.02305		3	20.162	0.02090
Mean	17.054	0.02187		Mean	20.325	0.02053
Std. Dev.	0.181	0.00160		Std. Dev.	0.216	0.00048
RSD (%)	1.060%	7.339%		RSD (%)	1.064%	2.357%

Table 7 (continued)

TLCA	Ret. Time (min)	Pk. Ht. (A.U.)		GLCA	Ret. Time (min)	Pk. Ht. (A.U.)
1	27.629	0.00687		1	38.15	0.01224
2	28.276	0.00807		2	38.647	0.01348
3	27.761	0.00811		3	37.913	0.01415
Mean	27.889	0.00768		Mean	38.237	0.01329
Std. Dev.	0.342	0.00070		Std. Dev.	0.375	0.00097
RSD (%)	1.226%	9.171%		RSD (%)	0.980%	7.292%

Table 8

Long Term Stability For Retention Time and Peak Height

TUDA	Ret. Time (min)	Peak Ht. (A.U.)		GUDA	Ret. Time (min)	Peak Ht. (A.U.)
1	4.506	0.02538		1	5.458	0.03863
2	4.504	0.02582		2	5.426	0.04002
3	4.507	0.02822		3	5.426	0.04300
4	4.706	0.02250		4	5.694	0.03351
5	4.703	0.02194		5	5.810	0.03386
6	4.397	0.02287		6	5.436	0.03389
7	4.479	0.02559		7	5.512	0.03781
Mean	4.543	0.02462		Mean	5.537	0.03725
Std. Dev.	0.117	0.00226		Std. Dev.	0.153	0.00365
RSD (%)	2.570%	9.182%		RSD (%)	2.767%	9.787%

TCA	Ret. Time (min)	Peak Ht. (A.U.)		GCA	Ret. Time (min)	Peak Ht. (A.U.)
1	7.028	0.01683		1	8.946	0.02988
2	7.018	0.01729		2	8.853	0.03213
3	7.015	0.01882		3	8.843	0.03338
4	7.452	0.01479		4	9.451	0.02588
5	7.502	0.01531		5	9.712	0.02490
6	6.903	0.01739		6	8.958	0.02933
7	7.060	0.01970		7	9.107	0.03330
Mean	7.140	0.01716		Mean	9.124	0.02983
Std. Dev.	0.236	0.00175		Std. Dev.	0.333	0.00342
RSD (%)	3.305%	10.211%		RSD (%)	3.648%	11.459%

Table 8 (continued)

TCDA	Ret. Time (min)	Peak Ht. (A.U.)		TDCA	Ret. Time (min)	Peak Ht. (A.U.)
1	12.754	0.01058		1	15.095	0.00930
2	12.728	0.01130		2	15.029	0.00995
3	12.683	0.01194		3	14.969	0.01059
4	13.778	0.00857		4	16.331	0.00736
5	14.000	0.00926		5	16.586	0.00758
6	12.605	0.01313		6	14.889	0.01155
7	12.917	0.01504		7	15.244	0.01315
Mean	13.066	0.01140		Mean	15.449	0.00993
Std. Dev.	0.573	0.00223		Std. Dev.	0.702	0.00208
RSD (%)	4.388%	19.546%		RSD (%)	4.545%	20.953%

GCDA	Ret. Time (min)	Peak Ht. (A.U.)		GDCA	Ret. Time (min)	Peak Ht. (A.U.)
1	16.853	0.01785		1	20.065	0.01521
2	16.624	0.01905		2	19.779	0.01616
3	16.552	0.01939		3	19.670	0.01663
4	18.084	0.01458		4	21.630	0.01302
5	18.686	0.01350		5	22.348	0.01143
6	16.982	0.02004		6	20.242	0.02070
7	17.260	0.02251		7	20.574	0.01998
Mean	17.292	0.01813		Mean	20.615	0.01616
Std. Dev.	0.801	0.00315		Std. Dev.	1.006	0.00338
RSD (%)	4.635%	17.351%		RSD (%)	4.878%	20.916%

TLCA	Ret. Time (min)	Peak Ht. (A.U.)		GLCA	Ret. Time (min)	Peak Ht. (A.U.)
1	27.860	0.00518		1	37.474	0.00906
2	27.810	0.00524		2	37.028	0.00928
3	27.551	0.00578		3	36.337	0.00976
4	30.585	0.00468		4	40.810	0.00766
5	31.140	0.00446		5	42.371	0.00641
6	27.629	0.00687		6	38.150	0.01224
7	28.276	0.00807		7	38.647	0.01348
Mean	28.693	0.00575		Mean	38.688	0.00970
Std. Dev.	1.508	0.00129		Std. Dev.	2.165	0.00246
RSD (%)	5.257%	22.471%		RSD (%)	5.595%	25.367%

CHAPTER SEVEN

CONCLUSIONS

This modified method provided an easy and reliable means for the detailed analysis of glyco and tauro bile acid conjugates. The bile acid profiles obtained were consistent with published data (3,6,26). High reproducibility of the analytical data indicated that quantization of bile acids in bile using the method outlined in this work can be carried out with a high degree authenticity. The specificity of the HPLC assay was affirmed in several ways:

1. The retention's of the individual bile acids in authentic bile standards were proven to be reproducible and stable at constant pH.

2. The corresponding peaks of bile acids in bile and authentic bile acid standards were exactly overlapped.

3. The additional calculations of the retention time using the method offered by Zhang and co-workers (60) in cases of uncertainty were made.

The simplicity and brevity of the presented method, along with its high efficiency and well resolved chromatographic peaks made it be very advantageous, particularly for the analysis of gallbladder bile.

The presented method has been used successfully for bile acid analysis in human bile and is particularly well suited for those analytical determinations that require an easy, straight-forward, and rapid method that allows for many analyses per day. The use of UV detection is very popular due to the widespread availability and relative inexpensiveness of UV detectors. The significance of UV detection in bile acid analysis, therefore, still means a great deal.

The analytical procedures applied to bile acid studies is typically very sophisticated because of difficulties of isolation, separation, and determination of the components in a multi-constituent mixture of a bile acid fluid. That is why a well chosen combination of convenient experimental conditions and a method of detection that provides a reliable and fast results, is the most important goal for these types of studies. The described method represents one of such approach that was successfully achieved. The bile acid study of this kind deserves further investigations to improve the equipment used and to increase the number of analytes accessible for determination.

In comparison of this method with that of Chi-Pui Pang and co-workers(3) it can be seen that in both methods the same type of solid phase extraction sample preparation technique, using commercially available, pre-packed octadecyl cartridges was used, but it was necessary to use a different internal standard. Many methods have used dexamethasone acetate, but it was found to be inappropriate for this method, since at the changed work parameters the peak for dexamethasone acetate was totally superimposed with the taurocholic acid peak. The steroid compound that was used for a bile acid assay by a group of German researchers, was found to be much more useful as an internal standard (22).

An isocratic mobile phase was used as it was in the original method but instead of 75% (v/v) methanol in phosphate buffer, 65% (v/v) composition of the same reagents was applied. The concentration and pH of the phosphate buffer was also changed. In the original method the buffer concentration was 15 mM and the pH was 6.25. In the modified method the buffer concentration was increased to 50 mM while the pH was decreased to 4.10. The flow rate in the original study initially was 0.5 mL/min. and then it was increased to 0.65 mL/min. in 1 minute linearly. In the modified study, it was found to be more convenient to use a steady but increased flow rate of 1.5 mL/min. The use of the more highly sensitive Programmable UV detector instead of a Diode-array detector permitted avoidance of degassing before and during chromatography.

In comparison of two methods it is easy to see that the older one provides better retention time, whereas the new one shows better resolution and the latter advantage is more important because it provides better accuracy as reflected in the analytical results.

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